

## Original Articles

The role of complement in *Streptococcus pneumoniae*-associated haemolytic uraemic syndromeÁgnes Szilágyi<sup>1,†</sup>,Nóra Kiss<sup>1,†</sup>,Csaba Bereczki<sup>2</sup>,Gyula Tálosi<sup>2</sup>,Katalin Rácz<sup>2</sup>,Sándor Túri<sup>2</sup>,Zsuzsa Györke<sup>3</sup>,Edina Simon<sup>4</sup>,Eszter Horváth<sup>4</sup>,Kata Kelen<sup>5</sup>,György S. Reusz<sup>5</sup>,Attila J. Szabó<sup>5</sup>,Tivadar Tulassay<sup>5</sup>and Zoltán Prohászka<sup>1</sup>

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## ABSTRACT

**Background.** Atypical forms of haemolytic uraemic syndrome (aHUS) include HUS caused by defects in the regulation of alternative complement pathway and HUS linked to neuraminidase-producing pathogens, such as *Streptococcus pneumoniae*. Increasing data support a pathogenic role of neuraminidase in the development of *S. pneumoniae*-associated haemolytic uraemic syndrome (SP-HUS), but the role of complement has never been clarified in detail. Therefore, we aimed to investigate whether the pathologic complement profile and genetic risk factors of aHUS are present in patients with SP-HUS.

**Methods.** Enrolling five patients with SP-HUS classical and alternative pathway activity, besides C3, C4, factors H, B, I and anti-factor H autoantibody levels were determined. The coding regions of *CFH*, *CFI*, *CD46* (*MCP*), *THBD*, *C3* and *CFB* genes were sequenced and the copy number of *CFI*, *CD46*, *CFH* and related genes were also analyzed.

**Results.** We found that in the acute phase samples of SP-HUS patients, complement components C4, C3 and activity of the classical and alternative pathways were decreased, indicating severe activation and complement consumption, but most of these alterations normalized later in remission. Three of the patients carried mutations and risk haplotypes in complement-mediated aHUS associated genes. The identified

mutations include a previously published *CFI* variant (P50A) and two novel ones in *CFH* (R1149X) and *THBD* (T44I) genes.

**Conclusions.** Our results suggest that severe complement dysregulation and consumption accompany the progress of invasive pneumococcal disease (IPD)-associated SP-HUS and genetic variations of complement genes may contribute to the development of this complication in a proportion of the affected patients.

## INTRODUCTION

Haemolytic uraemic syndrome (HUS) belongs to thrombotic microangiopathies (TMAs) and is defined by haemolytic anemia, thrombocytopenia and renal failure [1]. In its typical, diarrhoea-associated form, HUS is caused by exotoxins of certain bacteria, most frequently enterohaemorrhagic *Escherichia coli* [2]. In contrast, atypical forms of HUS (aHUS) include HUS caused by defects in the regulation of the alternative complement pathway on vascular endothelial cells [3] and HUS linked to neuraminidase-producing pathogens, such as *Streptococcus pneumoniae* [4] and influenza A [5]. In addition, drug-mediated or disease-associated forms, collectively classified as secondary HUS, have also been reported (for classification of TMA forms see [6]).

*Streptococcus pneumoniae* (pneumococcus) is a Gram-positive, encapsulated bacterium and a leading cause of pneumonia, meningitis and septicaemia in children and the elderly. Its asymptomatic nasopharyngeal colonization is common; nevertheless, it may cause severe illnesses and in cases, with life-threatening diseases such as septicaemia, pneumonia with pulmonary abscess or meningitis, it is referred to as invasive pneumococcal disease (IPD).

HUS is an uncommon complication of IPD, its prevalence is highest in children under 2 years of age, and this association is extremely rare in adults. The pathogenesis of *S. pneumoniae*-associated HUS (SP-HUS) has recently been reviewed by Copelovitch and Kaplan [7]. There is evidence of a role for neuraminidase of *S. pneumoniae* cleaving *n*-acetyl neuraminic acid from cell surfaces and exposing the Thomsen-Friedenreich (T) cryptantigen. Naturally occurring IgM anti-T antibodies bind to the exposed T antigen leading to red blood cell (RBC) agglutination, haemolysis, microvascular thrombosis, thrombocytopenia and the clinical picture of HUS [8]. There are several recent observations supporting the pathogenic role of neuraminidase in the development of SP-HUS, including development of HUS after infections with other neuraminidase-producing microbes such as influenza A virus [5] and *Capnocytophaga canimorsus* [9, 10].

As presented by Huang et al. [11], neuraminidase activity may be present in ~50% of patients with IPD, but only a minority of IPD patients progress to SP-HUS. The mechanism of SP-HUS development, particularly the host-related risk factors, is only scarcely known. Although an increasing number of genetic variants of certain complement genes are linked to the development of aHUS, a detailed investigation of the complement profile with the analysis of different variants

of the complement genes has never been published in the context of SP-HUS. We hypothesized that in some cases, a pathologic complement profile may be present in the acute phase of SP-HUS and some of the previously identified genetic risk factors of aHUS (such as mutations, risk haplotypes and copy-number variations of alternative pathway regulators) leading to complement alternative pathway dysregulation may contribute to the development of SP-HUS. Accordingly, a detailed investigation of the complement system was accomplished in a series of five patients with SP-HUS.

## MATERIALS AND METHODS

### Patients and samples

Five consecutive patients with SP-HUS were prospectively enrolled in this single-research laboratory-based investigation since August 2007, providing diagnostic services (ADAMTS13 and complement measurements) for patients suspected to have TMA in Hungary. The patient enrollment was closed in March, 2012.

Inclusion criteria: presence of HUS, according to the Center for Disease Control's definition: evidence of microangiopathic haemolytic anaemia; renal injury was defined if the following were present with acute onset: proteinuria (>0.5 g/24 h) and elevated creatinine (>88.4 µmol/L or >50% increase above baseline); and thrombocytopenia (<150 G/L) at presentation, or within 7 days of onset; and presence of invasive *S. pneumoniae* infection. Exclusion criteria: presence of disseminated intravascular coagulation or presence of severe comorbidities. Detailed description of enrolled cases is provided in Supplementary Material and in Table 1. As control, DNA samples of 100 healthy blood donors (aged 27–58, 60% female) were analysed.

Blood samples (EDTA-anticoagulated blood, sodium-citrate anticoagulated plasma and native serum) were taken by venipuncture or from central catheter before the initiation of plasma therapy (except for case 1). Cells and supernatant were separated by centrifugation, aliquots were made and stored at ≤70°C until determinations. An acute phase blood sample in this study refers to the first available blood sample of the patient, taken at the time when SP-HUS developed and the patient was transferred to the tertiary care centre.

### Determination of complement parameters, ADAMTS13 and neuraminidase activity

Functional assessment of the alternative pathway was done with the Wieslab AP ELISA kit [12], total classical pathway activity by a sheep-erythrocyte haemolytic test, C3 was measured with immunoturbidimetry, factor H antigen by sandwich-ELISA, factors C4, B and I with radial immune diffusion, IgG anti-factor H autoantibodies by direct ELISA. ADAMTS13 activity levels were determined using the fluorogenic substrate FRET-VWF73 (Peptides International). Details of the above laboratory determinations have been described elsewhere [13].

**Table 1. Admission laboratory results and disease course in patients with SP-HUS**

Variable	Case 1	Case 2	Case 3	Case 4	Case 5
Registry code	HUN49	HUN67	HUN129	HUN156	HUN274
Age (months)	12	11	37	18	29
Gender	Female	Female	Female	Female	Female
Vaccination	Prevenar	Pneumovax	Prevenar	Pneumovax	Prevenar
Pneumococcus-related disease	Pneumonia, empyema	Pneumonia, empyema	Pneumonia, empyema	Pneumonia, empyema	Pneumonia, pulmonary abscess
Evidence of Streptococcus infection	Pleural effusion, culture	Pleural effusion, culture	Pleural effusion, antigenic test	Pleural effusion, antigenic test	Pleural effusion, antigenic test
Length of anamnesis (days) <sup>a</sup>	5	14	7	10	8
Pneumococcus HUS case definition	Definite	Definite	Definite	Definite	Definite
Haemoglobin (g/L) <sup>b</sup>	67 (110–140)	83 (108–128)	96 (100–600)	41 (100–600)	39 (108–156)
Platelet count (G/L) <sup>b</sup>	65 (169–358)	73 (120–350)	33 (130–450)	33 (130–450)	25 (286–509)
Fragmentocytes	Yes	Yes	Yes	Yes	Yes
Direct Coombs' test	Positive	Positive	Positive	Positive	Positive
Serum neuraminidase activity	Positive	Positive	Positive	Positive	Positive
LDH (U/L) <sup>b</sup>	4313 (<850)	1729 (150–850)	7610 (200–600)	8004 (200–600)	6843 (310–790)
Creatinine (μmol/L) <sup>b</sup>	84 (27–62)	47 (18–36)	207 (70–90)	273 (70–90)	301 (21–36)
Fibrinogen (g/L) <sup>b</sup>	5.0 (2.0–4.5)	1.9 (2.0–4.0)	6.2 (1.5–4.0)	2.6 (1.5–4.0)	3.5 (2.4–5.0)
D-dimer (μg/mL) <sup>b</sup>	>4 (<0.5)	nd	>20 (<0.5)	nd	nd
RBC transfusion (unit)	22	4	6	4	2
Platelet transfusion (unit)	–	2	–	8	–
Plasma therapy (number of sessions)	PE (6)	FFP infusion (3)	PE (4)	–	–
Dialysis (days)	23	HF (3 sessions) HD (2 sessions)	PD (2)	–	PD(4)
Comorbidities	–	–	–	–	–
Duration of hospital stay (days)	101	11	25	15	30
Outcome (follow-up months)	Severe intracranial haemorrhage on hospital day 30, exitus on day 101	Fatal intracranial haemorrhage on hospital day 11 with exitus letalis	No sequelae (24)	No sequelae (18)	No sequelae (8)

FFP, fresh frozen plasma; HD, haemodialysis; HF, haemofiltration; nd, not determined; PD, peritoneal dialysis; PE, plasma exchange.

<sup>a</sup>Length of anamnesis was defined as the number of days from the first signs of infection until hospitalization due to HUS.<sup>b</sup>Reference ranges are indicated in parentheses.

Neuraminidase activity was kinetically determined in serum samples using a 2'-(4-methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid fluorogenic substrate [14] in phosphate-buffered saline, pH 5.5, 2 mM  $\text{CaCl}_2$  in white 384-well microplates with a Hidex Chameleon II microplate reader. Fluorescence ( $\lambda_{\text{ex}}$  360 nm/ $\lambda_{\text{em}}$  445 nm) over time was plotted and patient curves were compared with healthy control samples and mixed normal human serum. Serum neuraminidase activity was considered semiquantitatively positive if the reaction slope (patient sample) was at least double that obtained in the control samples.

### Molecular genetic analysis

Screening for mutations was carried out by DNA sequencing following PCR amplification of coding exons and flanking regions. The whole coding region of genes encoding complement factor H (*CFH*; MIM# 134370), factor I (*CFI*; MIM# 217030), membrane cofactor protein (*CD46*; MIM# 120920), thrombomodulin (*THBD*; MIM# 188040), factor B (*CFB*; MIM# 138470) and C3 (*C3*; MIM#120700) was analysed. Primer sequences and PCR conditions are available upon request. Following treatment with exonuclease I and alkaline phosphatase amplification products were processed for sequencing applying BigDye v3.1 sequencing chemistry (Applied Biosystems, Foster City, CA) and sequenced using an ABI 3130xl Genetic Analyser (Applied Biosystems). Sequencing chromatograms were evaluated applying CLC DNA Workbench 6.5 (CLC Bio, Aarhus, Denmark). Polymorphic variants were numbered from the A of the ATG translation initiation site as +1. Previously identified and nonsense mutations were accepted as pathogenetically relevant variations, while novel missense variants were regarded as mutations if they were not found in 100 healthy Hungarian controls (200 chromosomes) and international databases (dbSNP ([www.ncbi.nlm.nih.gov/snp](http://www.ncbi.nlm.nih.gov/snp)); Exome Variant Server [NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: <http://evs.gs.washington.edu/EVS/>) (Nov 2012 accessed)]. A possible functional effect of novel missense variations was predicted *in silico* using PolyPhen [15], PROVEAN [16] and MutationTaster [17].

In order to study copy-number alterations of selected complement genes, multiplex ligation-dependent probe amplification (MLPA) was performed with SALSA MLPA probemixes P236-A3 and P296-A1 (MRC-Holland, Amsterdam, the Netherlands) following the manufacturer's instructions. The P236-A3 mix is designed to detect deletions or duplications in the chromosomal region of complement factor H (*CFH*) and related genes (*CFHR1*, *CFHR2*, *CFHR3*, *CFHR5*), while the P296-A1 probemix contains probes specific for the genes encoding complement factor I (*CFI*) and MCP (*CD46*).

### Statistical analysis

Pair-wise linkage disequilibrium of *CD46* polymorphisms was calculated using Haploview 4.2 [18] based on genotype data of the CEU population (Caucasians of Northern and Western European descent) from the International HapMap Project ([www.hapmap.org](http://www.hapmap.org)).

## RESULTS

### Case definitions, diagnosis and follow-up

Five girls aged <38 months who met the Centre for Disease Control's definition of HUS [19] were included in our study (for detailed description of cases, see Supplementary Material). Furthermore, the patients were classified as having definite SP-HUS, based on the modified criteria presented by Copelovitch and Kaplan [4], in detail, evidence of HUS, evidence of invasive *S. pneumoniae* infection and exclusion of disseminated intravascular coagulation (Table 1). All of them had pneumonia with effusion, empyema or pulmonary abscess, none of them had meningeal signs or meningitis and no comorbidities were present. Serum neuraminidase activity was positive in all cases and accordingly, the direct Coombs test was also positive in all cases (Table 1).

Two of the five patients died during hospital stay due to intracranial haemorrhage, whereas the other three patients were released without sequelae and had no disease recurrence during follow-up (Table 1).

### Complement profile

Table 2 shows the results of the complement testing in the acute admission, and in the remission (at least 2 months after hospital discharge) samples of the patients. Consumption of components C3 and C4 with decreased total classical (except case 1) and alternative pathway (AP) activity was the most characteristic alteration observed in all of the samples in the acute phase. Case 3 had strikingly low (deficient) alternative pathway activity. Complement factors B and H were within the reference range in acute samples, whereas cases 2 and 4 had moderately decreased factor I levels. In those cases with available remission samples, all of the above complement alterations seen in the acute phase returned to the reference range, except the slightly decreased C3 and factor H levels as well as AP activity of case 3 and decreased factor I levels of case 4. All of the patients were negative for anti-factor H autoantibodies and had moderately decreased ADAMTS13 activity in the acute phase (19–44%) that normalized later in remission.

### Molecular genetic analysis

To investigate genetic alterations in our cases, coding regions of *CFH*, *CFI*, *CD46* (*MCP*), *THBD*, *C3* and *CFB* genes were sequenced. As presented in Table 3, mutations were identified in three cases. Case 3 carried a heterozygous transition (c.3445C>T) that leads to the creation of a stop codon in SCR 19 at position 1149 (Arg1149X) expectedly causing premature termination of complement factor H translation. In accordance with this assumption, the complement factor H level of this patient was below lower reference limit in remission (Table 2). Case 4 presented a heterozygous transversion (c.148C>G) causing a proline-to-alanine change (Pro50Ala) in complement factor I. This mutation was previously described in two aHUS patients and was shown to result in reduced intracellular and secreted IF levels *in vitro* [20]. Accordingly, complement



**Table 2. Acute admission/remission (at least 2 months after hospital discharge) complement and ADAMTS13 values of patients with SP-HUS**

Variable (reference range)*	Case 1	Case 2	Case 3	Case 4	Case 5
Classical pathway activity (48–103 CH50/mL)	61/na	27/na	40/55	19/67	29/50
Alternative pathway functional activity (70–105%)	46/na	34/na	1/60	61/80	37/69
Complement C3 (0.9–1.8 g/L)	0.54/na	0.33/na	0.45/0.78	0.54/1.34	0.66/1.04
Complement C4 (0.15–0.55 g/L)	0.14/na	0.05/na	0.06/0.37	0.05/0.34	0.08/0.19
Complement factor B (70–130%)	99/na	97/na	89/87	76/125	87/90
Complement factor I (70–130%)	87/na	62/na	88/103	63/59	96/83
Complement factor H (127–447 mg/L)	340/na	131/na	139/106	315/303	372/245
Anti-factor H IgG autoantibody	Negative/na	Negative/na	Negative/negative	Negative/negative	Negative/negative
ADAMTS13 activity (67–151%)	44/na	24/na	19/81	19/111	32/86
na, not available.					

factor I level of case 4 was below the normal range in acute and remission phase as well (Table 2). Case 5 was found to be heterozygous for a cytosine-to-thymine substitution (c.131C>T), causing threonine to isoleucine change at codon 44 of thrombomodulin. This variation was not found in 100 healthy Hungarian controls and not reported by dbSNP or Exome Variant Server release ESP6500SI containing data from 6503 samples. The effect of this mutation was *in silico* predicted to be possibly damaging by PolyPhen (score 0.524), deleterious by PROVEAN (score –4.070) but polymorphism by MutationTaster.

Sequencing of complement genes revealed the presence of many polymorphic variants of which those, causing amino acid change or reported previously as risk or protective factors for developing aHUS, are presented in Table 3. As deduced from genotype data, one patient (case 3) carried the H3 risk haplotype of *CFH* gene that consists of among others the T allele of –331C/T, G allele of c.2016A/G (Q672Q) and T allele of c.2808G/T (E936D) polymorphisms, reported as risk alleles for aHUS in several studies [21, 22]. Three patients carried risk alleles of MCP polymorphisms previously described to be associated with aHUS that are –547G, –261G, IVS823G, IVS9–78A and IVS1243C [21, 23]. Two constituents (rs859705 (IVS12638A/G) and rs7144 (c.2232C/T)) of the so-called MCPggaac haplotype were not determined in our patients; however, linkage analysis—applying data of the International HapMap Project—showed that these are strongly linked to rs1962149 (IVS9–78G/A), hence cases 3, 4 and 5 carrying risk alleles are strongly supposed to bear the MCPggaac aHUS risk haplotype in heterozygous form.

To reveal deletions or duplications that may influence disease development, *CFI*, *CD46* (MCP), *CFH* and its related genes were studied applying MLPA probemixes of MRC-

Holland. None of the patients showed copy-number alterations in *CFI*, *CD46* and *CFH* genes, while three (cases 2–4) were heterozygous carriers of a common deletion of *CFHR1* and *CFHR3* genes.

## DISCUSSION

To the best of our knowledge, this is the first study thoroughly investigating the complement system in patients with SP-HUS. In the acute phase of SP-HUS components and activity of the classical and alternative pathways were decreased, indicating severe activation and consumption of complement, while most of these alterations normalized later in remission. In addition, three of the five SP-HUS patients carried mutations and/or risk haplotypes in genes previously reported to associate with complement-mediated aHUS. Two of the identified mutations (the known factor I variation and the new factor H mutation causing stop codon) can be considered as functionally relevant, whereas the functional effect of the third novel mutation in the thrombomodulin gene is unknown. Based on these observations, we conclude that severe complement dysregulation and consumption, in addition to neuraminidase action, accompany the progress of IPD-associated SP-HUS and genetic variations of complement genes may contribute to the development of this complication in a proportion of the affected patients.

Neuraminidase A, a major determinant of pneumococcal adherence to epithelial cells [24], is produced by virtually all strains of *S. pneumoniae* [25]. The T-antigen is a disaccharide that forms the core structure of O-linked mucin-type glycans and is a cryptic antigen normally hidden by terminal sialic acid residues [26]. It was only recently proven that T-antigen

**Table 3. Genetic analysis of patients with SP-HUS**

		Case 1	Case 2	Case 3	Case 4	Case 5
	Affected gene	None	None	<i>CFH</i>	<i>CFI</i>	<i>THBD</i>
Mutations <sup>a</sup>	Numbering from Met1	–	–	Arg1149X	Pro50Ala	Thr44Ile
	Numbering based on the mature protein	–	–	Arg1131X	Pro32Ala	Thr26Ile
	Reference	–	–	Novel	Bienaime <i>et al.</i> [20]	Novel
Missense variations <sup>a,b</sup>	<i>CFB</i>	R32W (rs12614)	R32W (rs12614)	R32W (rs12614)	G252S (rs4151651)	–
	<i>CFH</i>	Y402H (rs1061170)	–	E936D (rs1065489)	–	Y402H (rs1061170)
aHUS risk haplotypes <sup>a,c</sup>	<i>CFH</i>	–	–	<i>CFH</i> H3	–	–
	<i>CD46</i>	–	–	MCPggaac	MCPggaac	MCPggaac
Copy-number variations <sup>a</sup>	<i>CFI</i>	–	–	–	–	–
	<i>CD46</i> (MCP)	–	–	–	–	–
	<i>CFH</i>	–	–	–	–	–
	<i>CFHR1</i>	–	Deletion	Deletion	Deletion	–
	<i>CFHR2</i>	–	–	–	–	–
	<i>CFHR3</i>	–	Deletion	Deletion	Deletion	–
	<i>CFHR5</i>	–	–	–	–	–

<sup>a</sup>Each of the denoted genetic alterations was carried in heterozygous form.  
<sup>b</sup>Polymorphisms (revealed by sequencing of *CFH*, *CFB*, *CFI*, *CD46*, *THBD* and *C3* genes) causing amino acid changes are listed. No other non-synonymous variant occurred in patients.  
<sup>c</sup>Presence of previously reported aHUS risk haplotypes was based on the simultaneous carriage of their constituents [*CFH*: –331T (rs3753394), c.184G (rs800292), c.1204T (rs1061170), c.2016G (rs3753396), c.2808T (rs1065489); *CD46*: c.–547G (rs2796267), c.–261G (rs2796268), IVS1–156G (rs2724384), IVS4249delA (rs34743953), IVS823G (rs2724374), IVS9–78A (rs1962149), IVS1243C (rs11118580)].

exposure in *S. pneumoniae* infection is due to pneumococcal neuraminidase A [27]. However, the presentation of neuraminidase activity in *S. pneumoniae* infection is suggestive but not specific for HUS, as presented by Huang *et al.* [11]. In that study, neuraminidase activity was demonstrated in 100% of SP-HUS patients, but also in 67 and 43% of patients with pneumococcus-associated anemia and uncomplicated IPD, respectively. It is important to note that serum neuraminidase activity and direct Coombs positivity were present in all of our five patients. These observations indicate that in the setting of IPD, neuraminidase activity is required but not sufficient to initiate HUS and additional, as yet unidentified factors are also present.

Atypical HUS (i.e. HUS in the absence of associating disease, Shiga-like toxin or pneumococcus) appears to have a genetic basis with identified mutations in ~60–65% of cases. Loss-of-function mutations in genes encoding complement regulatory proteins like factor H, MCP, factor I or thrombomodulin have been demonstrated in 20–30%, 5–15%, 4–10%

and 3–5% of patients, respectively, whereas gain-of-function mutations in genes of C3 convertase proteins, C3 and factor B, in 2–10% and 1–4% [3]. In addition, 6–25% of patients have anti-factor H antibodies [3, 28]. We have identified three mutations in five SP-HUS patients in our series in genes previously linked to aHUS (1 in factor H, 1 in factor I and 1 in thrombomodulin gene). The functional role of two identified mutations is apparent, since one is a nonsense substitution in *CFH* and another is a missense variation in *CFI* previously linked to decreased factor I level *in vitro* and reported to be present in aHUS patients but not in healthy controls [20]. The thrombomodulin variation (considered as a novel mutation, since it was not present in databases and has not occurred in healthy controls in our study) was not reported previously, and its functional consequences was not yet analyzed. However, it should be noted that *in silico* prediction provided possible functional consequences. Furthermore, this variation is located in the lectin-like domain of thrombomodulin, where two aHUS-associated mutations (Ala43Thr and Asp53Gly)

have already been reported which are less effective in enhancing factor I-mediated conversion of C3b to iC3b on the cell surface after complement activation *in vitro* [29]. Therefore, it is tempting to hypothesize that this novel mutation causing threonine to isoleucine change at codon 44 may also disturb this function of the protein, but functional studies are needed to confirm this hypothesis.

The most prominent pathological sign in the complement profile of SP-HUS patients was the strong activation and consumption of classical and alternative pathways, as reflected by low levels of CH50, C3, C4 and total alternative pathway activity. Whether these alterations can be utilized for diagnostic, prognostic or even therapeutic purposes in IPD or acute SP-HUS requires further studies; however, we strongly encourage the rapid testing of the complement profile in all forms of acute TMAs, including SP-HUS [30]. This complement profile agrees with the initial observations on decreased C3 and C4 levels in acute phase of SP-HUS by Johnson and Waters [31].

The severe IPD infection and consequential septicemia and bacteraemia may have contributed to the pathological complement profile in our patients. The activation and consumption of the alternative pathway during acute pneumococcal infections including pneumonia have been described [32, 33], but in these studies severe pneumococcal infection was not accompanied by consumption of classical pathway components. It was also suggested that *in vivo* depletion of AP factors is more pronounced in patients with complicated than with less severe pneumococcal disease [34]. However, the uniform alteration in classical pathway component C4 during acute pneumococcal infection is a novel finding in SP-HUS and has not been observed previously. Therefore, our observation on the consistently decreased C4 levels, indicating consumption of the classical pathway, seems to be indicative of the T-exposure and development of IPD-associated HUS. Exposure of T-antigen in the context of *S. pneumoniae* infection may enhance complement activation and consumption via multiple pathways. First, preformed anti-T IgM antibodies may bind to structures exposing T-antigens on different cells including RBCs, epithelial and endothelial cells resulting in the activation of the classical pathway (consumption of C4 and C3). In addition, loss of terminal sialic acids from glycans in response to neuraminidase action may lead to amplification of complement activation via the AP, since the major soluble regulator of the AP, factor H, binds to sialic acids [35] and functions as a cofactor for the factor I-mediated C3b cleavage (consumption of C3 and decrease in AP activity). Taken together, infection by *S. pneumoniae* as a trigger, subsequent loss of terminal sialic acids from host glycans together with genetic variants of complement regulators may collectively lead to dysregulated complement activation with consumption and development of SP-HUS in patients with IPD. The transient decrease of ADAMTS13 activity during the acute phase of SP-HUS was reported in a patient previously [36]. Here, we further strengthen this observation, since in all of our five patients decreased ADAMTS13 activity was detected during the acute phase of the disease, reflecting the ongoing microangiopathic process.

An interesting aspect of our results is related to the potential disease recurrence in SP-HUS. Since disease recurrence is a characteristic feature of complement-mediated aHUS, it is tempting to speculate that there is a risk of recurrence in SP-HUS as well, if the complement-related predisposition is significant in this disease. The number of reported SP-HUS cases with outcome and follow-up data in the literature is low (~100). It seems that the mortality during the first episode is high (~10%), furthermore, chronic kidney disease or end-stage renal disease affects other 20% of patients [7, 37–40]. However, for those reported with follow-up data, disease recurrence has not been reported until now. Further studies with aggregate analysis of published clinical data and reporting of long-term outcomes are necessary to estimate the risk of disease recurrence in SP-HUS.

It is important to note that all of our patients received vaccinations against pneumococcus (three patients conjugate, whereas two polysaccharide vaccines, Table 1). It has been suggested that the introduction of conjugate vaccines might have caused a dramatic decline in the incidence of vaccine-strain-linked diseases; however, an increase of non-vaccine strains was also observed, for example the emergence of strain 19A in the context of SP-HUS is well documented [4]. It is therefore tempting to speculate that non-vaccine strains with increased neuraminidase activity will more frequently cause severe invasive pneumococcal diseases, for example SP-HUS. Unfortunately, we do not have information on the serotype of the five strains causing SP-HUS in our patients. Furthermore, it is interesting to note that all of our patients were girls, but this is in contrast to the published literature where ~1:1 female-to-male ratio has been reported for SP-HUS patients; therefore, this predominance of females may be due to chance only.

There are particular strengths and limitations of our study. In the time period of patient recruitment for this study determination of the Thomsen–Friedenreich cryptantigen was unavailable in Hungary; therefore, we could not present such data. We were able to include only five patients into our series precluding to design a case–control analysis with statistical tests or to make group comparisons. However, all cases during a fixed time period were consecutively included in a prospective manner, and determinations of complement parameters and genetic analysis were completely done for them. Furthermore, all clinical and laboratory data to provide a precise diagnosis and classification of SP-HUS were available. Notwithstanding, at this moment our observations are to be considered as preliminary and hypothesis generating only.

## CONCLUSION

In conclusion, here we described a series of five patients with definitive SP-HUS and provided descriptive data of their complement profile and underlying genetic variations. All patients presented with detectable serum neuraminidase activity, severely activated and consumed classical and alternative pathways in the acute phase of disease. Two among the

five patients carried pathogenic mutations, while one carried a yet uncharacterized new mutation, besides, three had risk haplotypes in genes (complement factor H and membrane-cofactor protein) which have previously been reported in association with complement-mediated aHUS. These results strongly suggest that in a proportion of the affected patients, the same genetic variants predisposing to complement-mediated aHUS may contribute to the development of SP-HUS in the context of IPD as well.

## SUPPLEMENTARY DATA

Supplementary data are available online at <http://ndt.oxfordjournals.org>

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## CONFLICT OF INTEREST STATEMENT

None declared.  
Clinical Trial Registration: none.

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